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Potent toxins in Arctic environments – Presence of saxitoxins and an unusual microcystin variant in Arctic freshwater ecosystems

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A B S T R A C T

Cyanobacteria are the predominant phototrophs in freshwater ecosystems of the polar regions where they commonly form extensive benthic mats. Despite their major biological role in these ecosystems, lit tle attention has been paid to their physiology and biochemistry. An important feature of cyanobacteria from the temperate and tropical regions is the production of a large variety of toxic secondary metabo lites. In Antarctica, and more recently in the Arctic, the cyanobacterial toxins microcystin and nodularin (Antarctic only) have been detected in freshwater microbial mats. To date other cyanobacterial toxins have not been reported from these locations. Five Arctic cyanobacterial communities were screened for saxitoxin, another common cyanobacterial toxin, and microcystins using immunological, spectro scopic and molecular methods. Saxitoxin was detected for the first time in cyanobacteria from the Arctic. In addition, an unusual microcystin variant was identified using liquid chromatography mass spectrom etry. Gene expression analyses confirmed the analytical findings, whereby parts of the *sxt* and *mcy* operon involved in saxitoxin and microcystin synthesis, were detected and sequenced in one and five of the Arc tic cyanobacterial samples, respectively. The detection of these compounds in the cryosphere improves the understanding of the biogeography and distribution of toxic cyanobacteria globally. The sequences of *sxt* and *mcy* genes provided from this habitat for the first time may help to clarify the evolutionary ori gin of toxin production in cyanobacteria.

1. Introduction

Several phototrophic organisms survive the harsh climate of the high Arctic regions, including some higher plants, mosses, lichens, various algal groups and cyanobacteria. Cyanobacteria are globally distributed, but in high Arctic freshwater ecosystems they repre sent the dominant primary producers [1,2]. Special features such as resistance to ultraviolet (UV) radiation, freeze thaw cycle adap tation and nitrogen fixation allow their survival in these extreme environments [2]. During the polar summer, when both light and temperatures above the freezing point prevail, cyanobacterial com munities thrive. They develop highly diverse benthic or floating mats in freshwater streams, ponds and on soils continuously fed with melt water that can be several centimeters thick and extend over large areas [2,3]. These extensive mats form the basis of a small but diverse and dynamic ecosystem accommodating a vari ety of organisms such as nematodes, rotifers, tardigrades [4], mosses and moss infecting oomycetes such as the recently de scribed *Pythium polare* [5].

Saxitoxins (STXs) (Fig. 1A) are carbamate alkaloids, a group of fast acting neurotoxins, inhibiting neuronal signal propagation of most higher organisms [6]. They are typically produced by marine dinoflagellates [7]. However, planktonic and benthic cyanobacteria from temperate and tropical regions, e.g. *Aphanizomenon* spp., *Ana baena circinalis*, *Cylindrospermopsis raciborskii*, *Planktothrix* spp., and *Lyngbya wollei* [8], are also known to produce STXs. Microcystins (MCs) (Fig. 1B) on the other hand, represent a group of ⩾80 structural heptapeptide variants with varying hepato , renal , and

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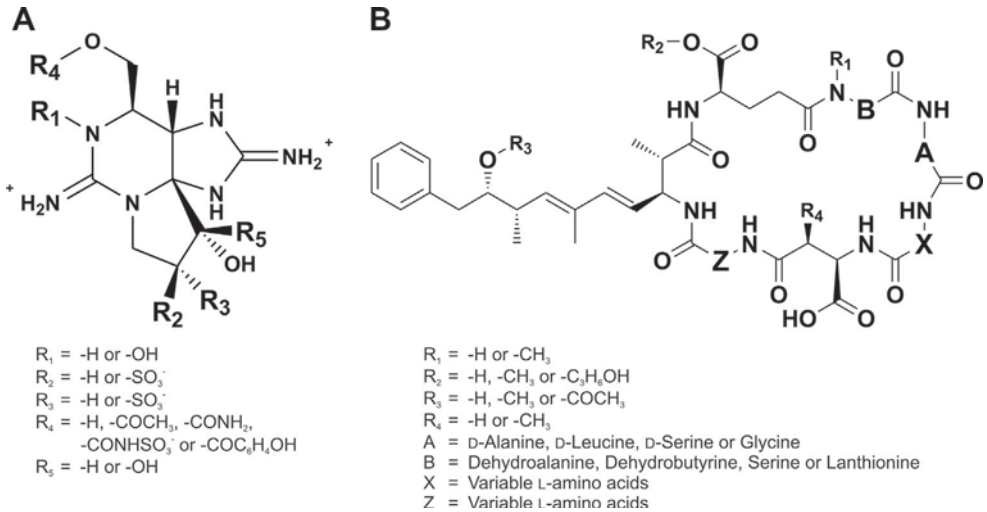


Fig. 1. Chemical structures of saxitoxin (STX) and microcystin (MC). General structure of the STXs (A); adapted from Humpage et al. [8] and the general structure of the MCs (B); adapted from Puddick [67].

neurotoxicity [9,10]. They appear to act primarily via specific inhi bition of serine/threonine phosphatases [8,10], thereby interfering with one of the most important regulatory mechanisms of the cell. MCs are produced by a large variety of planktonic and benthic cyanobacterial genera including *Microcystis*, *Nostoc*, *Planktothrix*, *Anabaena*, *Synechococcus* and *Snowella* [8,11].

The molecular basis for the production of both toxins are large and variable gene clusters, encoding enzymes involved in second ary metabolite production such as polyketide synthetases and/or non ribosomal peptide synthetases [12 14]. The distribution of these gene clusters among different strains of cyanobacteria does not necessarily correlate with the actual production of the toxins [15]. Therefore the presence of these genes in a given ecosystem is only an indication for the presence of the toxins and is therefore considered in this study as providing evidence for the 'potential to produce toxins'. These biosynthetic steps are energetically expen sive for cyanobacteria [16], and this has prompted considerable speculation on their ecological function. To date the physiological function and ecological regulation of both STXs and MCs are poorly understood [17 19]. The current hypotheses aiming to explain this relatively enormous investment of energy in the synthesis of these toxins, include protection against grazing pressure, UV radiation, and reactive oxygen species, as well as their function as signaling molecules in a quorum sensing like manner [16,19 22]. The devel opment of toxins as protection against grazers appears to be the least plausible hypothesis as the corresponding gene clusters ap pear to have been present in ancestral cyanobacteria species that have existed prior to the mesoproterozoic period [23 25], i.e. mil lions of years prior to the emergence of potential eukaryotic graz ers of cyanobacteria. However it cannot be excluded that new functions have developed in the course of evolution.

In view of the ancient origin and the high conservation of the toxin gene clusters it is not surprising that cyanobacteria inhabit ing remote and pristine areas, e.g. the rudimentary environments of the polar regions, could produce toxins. The presence of MCs in cyanobacterial mats has been reported for several locations in Antarctica [26 28], whereas this has only recently been demon strated for Arctic cyanobacteria from northern Baffin Island [29]. Kleinteich et al. [29] demonstrated that culturing of cyanobacterial mats in the laboratory at increased temperatures caused a marked rise in the concentration of MCs in concert with shifts in the diver sity of the cyanobacterial mat community composition. Whether the latter is a response to temperature stress, changing diversity of community structures or indeed a marker of a growth advantage of toxin producing cyanobacteria still needs to be ascertained. Saxitoxins, on the other hand, have never been reported in polar environments and cyanobacterial toxins in general remain under studied in this habitat.

In this study five cyanobacterial communities from the Arctic were screened for the presence of STX using enzyme linked immu nosorbent assay (ELISA) and further confirmation was undertaken using high performance liquid chromatography with fluorometric detection (HPLC FLD). Furthermore an unusual MC variant was identified using liquid chromatography mass spectrometry (LC MS). Samples were also screened for selected genes involved in the synthesis of MC and STX, thereby providing evidence of the toxin producing potential of Arctic cyanobacterial communities.

2. Materials and methods

2.1. Study sites and sampling

Five samples of cyanobacterial communities were collected during an expedition to northern Baffin Island in the vicinity of Cape Hatt (72°30′ N and 79°47′ W) in August September 2009 from microbial communities on wet soil, small streams and ponds (see Supplemental Fig. 1 for GPS coordinates). Samples for DNA extraction and toxin analysis were sealed in sterile tubes and those for RNA analysis immersed in RNAlater (Qiagen, Hilden, DEU). DNA and RNA samples were frozen (20 °C) within 24 h after collection and stored for approximately 6 months until further analysis.

2.2. Screening for saxitoxin and microcystin

2.2.1. Saxitoxin extraction

Frozen cyanobacterial material was lyophilized and their dry weight recorded. Samples for STX analysis were extracted as de scribed by Smith et al. [30]. Briefly, 50 mg of lyophilized material was homogenized in 5 mL H_2O using a mortar and pestle and dried under nitrogen flow. The dried material was dissolved methanol (4 mL) acidified with acetic acid (0.1%), vortexed (15 min), and placed in an ultrasonic water bath (15 min, ice cold). The suspen sion was centrifuged (30 min, 4000*g*) and the supernatant trans ferred into a separate tube for HPLC FLD analysis.

To ensure complete STX recovery from the residual pellet, 5 mL HCl (0.1 M) was added and the pellet boiled (5 min, 100 °C), directly followed by ultra sonication (10 min, ice cold). Cell debris was removed by centrifugation (30 min, 3000*g*), and the supernatant adjusted to pH 7.0 with NaOH. The supernatant was filtered through a 0.2 μm filter and pooled with the supernatant obtained in the first extraction. The pooled supernatant was dried under nitrogen gas flow and stored at −20 °C until HPLC FLD analysis.

2.2.2. Microcystin extraction

Lyophilized material (173 mg) was homogenized to a powder using a mortar and pestle. Methanol (75%, 5 mL) was added to the powder, the suspension placed in an ultrasonic water bath for 30 min (ice cold), centrifuged (30 min, 4000*g*), and the supernatant removed for further processing. The extraction was repeated three times. The supernatants were pooled, dried under nitrogen flow, re suspended in 15 mL H_2O, and loaded onto C18 cartridges (Sep Pak, Waters, Dublin, IRL). MC was eluted from the C18 cartridges with methanol (100%), the methanolic eluate dried under nitrogen gas flow, and resuspended in 3 mL methanol (20%). The extract was centrifuged (20 min, 13,000*g*) and the supernatant stored at −20 °C until further analyses.

2.2.3. Saxitoxin analysis

The STX (PSP) ELISA kit (ABRAXIS, Warminster, USA) was employed to analyze for STX according to the manufacturer's protocol. This STX (PSP) ELISA has a limit of detection (LOD) of 0.0215 ng/mL. Analytical data were generated based on three independent replicate analyses each with duplicate technical replicates.

Saxitoxins were also analyzed using HPLC FLD as described Smith et al. [30]. This HPLC detection method had a LOD of 0.1 mg/kg total STXs.

2.2.4. Microcystin analysis

The extract of cyanobacterial sample A, which had previously tested positive for MC by ADDA ELISA [29], was analyzed by LC MS in order to identify the MC congener/s present. LC MS was undertaken on a HPLC system (UltiMate 3000; Dionex) coupled to an AmaZon X (Bruker Daltonics) electrospray ionization ion trap mass spectrometer (ESI IT MS^n). Samples (20 μL) were separated on a C_{18} column (Ascentis Express C_{18}, 100 × 2.1 mm, 2.7 μ; Supleco Analytical) using a gradient system of 98% H_2O + 0.1% formic acid (v/v; solvent A) and 98% acetonitrile + 0.1% formic acid (v/v; solvent B) with the following gradient program; the sample was loaded in 10% B; 10% B was held for 1 min and increased to 100% B over 12 min; 100% B was held for 2 min; the solvent concentration was returned to 10% B in 1 min and the column re equilibrated for 4 min. The eluting compounds were transferred into the IT MS using a capillary voltage of 3.5 kV and a nebulizer pressure of 3.0 bar. Desolvation was accomplished with a nitrogen flow of 8 L/min at 220 °C. Tandem MS (MS/MS) spectra were gathered using the doubly or singly protonated ions of the target compounds and collision induced dissociation (CID) to induce fragmentation of the parent ion (collision amplitude of 1.0).

A recently developed thiol derivatization technique was used to determine whether the position seven amino acid in the MC observed was *N* methyldehydroalanine (Mdha) or dehydrobutyrine (Dhb) [31]. Sample A (1420 μL) was mixed with 200 mM $NaHCO_3$ (pH 9.7; 360 μL) in a septa capped vial and left to equilibrate to 30 °C. Following LC MS injection of the original extract, β mercaptoethanol (20 μL) was added to the extract of sample A and the vial inverted to mix. The reaction mixture was maintained at 30 °C in the sample tray of the LC MS apparatus and injections were made periodically over a 90 h period.

2.3. Screening for genes involved in toxin synthesis

2.3.1. Nucleic acid extraction

DNA was extracted from 5 10 mg of frozen material using the MO BIO PowerSoil® DNA Isolation Kit following the manufacturer's recommendations. Due to the heterogeneity of the sample material, three individual extractions were performed and the pooled extracts used for downstream applications. RNA was extracted from 5 to 10 mg of material stored in RNAlater (Qiagen, Hilden, DEU). RNAlater was removed by patting the material on a dry stack of paper, and RNA extracted using the MO BIO PowerBiofilm® RNA extraction kit following the manufacture's protocol. RNA was eluted with RNase free water and stored at −80 °C.

2.3.2. Detection of genes involved in toxin synthesis

PCRs targeting the *mcy* and *sxt* operon for MC and STX synthesis respectively were performed with primer pairs and at annealing temperatures as listed in Supplemental Table 2; primers were from MWG eurofins (Ebersberg, DEU). For the reactions either the Master Mix™ (Fermentas, St. Leon Rot, DEU) or the Phusion™ polymerase mix (NEB, Ipswich, USA) was used supplemented with BSA, DMSO and $MgCl_2$. Bands were excised from a 1.5% agarose gel (TAE) using a sterile scalpel, purified with a gel extraction kit (Fermentas, St. Leon Rot, DEU) and sequenced bi directionally using the primers listed in Supplemental Table 1 at MWG eurofins (Ebersberg, DEU). Messenger RNA of the *sxt* operon was reverse transcribed into cDNA using gene specific reverse primers (Supplemental Table 2) and a standard protocol for reverse transcription (20 U RNAse Inhibitor, 0.8 mM dNTPs, 7 μL of extracted RNA, 70 U M MuLV). Enzyme and chemicals for RT PCR were from NEB (Ipswich MA, USA). The cDNA produced was used as template for PCR as described above. *Microcystis aeruginosa* CCAP 1450/16 served as a positive control for *mcy* genes, but no positive control of cyanobacterial origin was available for the *sxt* genes. The obtained sequences were analyzed using Geneious™ software (Geneious Pro 5.3.6) and the closest matches identified using NCBI's BLAST tools (mega BLAST and BLASTn). Phylogenetic trees using *sxtA* sequences were built using the Geneious™ tree builder (Jukes Cantor, Neighbour joining method). The obtained 657 bp product of the *sxtA* gene was deposited in the GenBank database under the accession JX887897 (Supplemental Table 3). The obtained 128 bp product of the *sxtA* gene is displayed in Supplemental Table 5 since GenBank does not allow deposition of sequences shorter than 200 bp.

2.4. Identification of toxin producer/s

2.4.1. Cloning of 16S rRNA and intergenic spacer region (ITS)

Two samples that returned positive results from toxin analyses were selected for construction of 16S rRNA gene and *intergenic spacer region* (ITS) region clone libraries. Amplification was achieved with the cyanobacteria specific primer pair 27F and 23S30R (Supplemental Table 2) in a 50 μL PCR reaction (4 min, 95 °C; 35 × [92 °C, 60 s; 55 °C, 60 s; 72 °C, 120 s]; 72 °C, 10 min) containing the Fermentas (St. Leon Rot, DEU) Master Mix, 2.5 mM $MgCl_2$, 0.2 μg/μL BSA, 3% DMSO and 0.5 μM of each primer. The PCR products were separated on a TAE 1.5% agarose gel and the bands excised using a sterile scalpel. After purification with the GeneJET™ Gel Extraction Kit (Fermentas, St. Leon Rot, DEU) PCR products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, USA) following the standard protocol with a DNA to vector ratio of four to one. For each sample approximately 40 clones were retrieved and conserved in sterile TE buffer. Each clone insert was amplified in a PCR reaction (95 °C, 4 min; 35 × [92 °C, 30 s; 52 °C, 30 s; 72 °C, 70 s]; 72 °C, 5 min) using the Fermentas PCR Master Mix (St. Leon Rot, DEU), 0.25 μM of each TOPO primer

M13, 2 mM $MgCl_2$, 3% DMSO and 0.3 μL of DNA template. Products were subjected to a Restriction Fragment Length Polymorphism (RFLP) analysis using restriction enzymes AluI and ScrF1 (NEB, Ipswich, USA) (37 °C, 2 h) and subsequently visualized on an agarose gel (TAE, 2.5% agarose) in order to identify groups of multiple clones of the same phylotype. Two to three representative clones of each individual RFLP pattern (phylotype) were selected and sequenced at GATC Biotech, Konstanz, Germany using the TOPO primers T3 and T7 as well as the 16S rRNA gene specific primers 27F, 359F and 23S30R for verification. The resulting sequences were analyzed using Geneious™ software (Geneious Pro 5.3.6) and their closest matches identified via a Mega BLAST search of the GenBank database. Sequences were deposited in GenBank under the accession numbers as indicated in Supplemental Table 3.

2.4.2. Morphological identification

Microscopic analysis was performed using a Nikon Eclipse TS 100 Microscope and images documented with a Nikon Digital Sight DS 5M camera. Image quality was improved using XnView for Windows Software (version 1.97.6; Libformat version 5.70) and scale bars included by Corel Photo Paint 11 for Windows (version 11.633). Species identification down to genera level was made using the taxonomic guides of Komarek and Anagnostidis [32,33].

2.5. Data evaluation

Data were evaluated using Graphpad Prism™ Software (Prism 5 for Windows, Version 5.04).

3. Results

3.1. Detection of toxins in Arctic cyanobacterial communities

3.1.1. Saxitoxin detection

One of the five samples analyzed with the STX ELISA tested positive (Sample E; 21 (SD = 16) μg STX/kg dry weight; $n = 6$) well above the detection limit of the STX ELISA and in the center of the standard curve. However, the concurrent HPLC FLD analyses were not able to confirm this finding, possibly due to the detection limit of the individual STX variants (LOD between 0.5 and 13 μg/kg). Additionally different sample matrices may result in matrix suppression which may increase limits of detection (Pers. comm. Michael Boundy, Cawthron, October 2012). None of the other samples tested positive in the STX ELISA, either as original samples or when cultured in the laboratory at various temperatures (see Kleinteich et al. [29] for laboratory culture conditions). Subsequent PCR amplification of two segments (128 bp and 657 bp) of the *sxtA* gene provided for a positive signal in sample E but not for the other four samples (Table 1). Additionally, the *sxtA* gene mRNA was detected in sample E (Fig. 2).

The *sxtA* gene encodes for a polyketide synthetase, which is part of the recently identified cyanobacterial *sxt* gene cluster [34]. The amplified 128 bp and 657 bp products of sample E were sequenced and compared to the GenBank data base. The 657 bp gene segment shared a high similarity with known *sxtA* genes from the freshwater cyanobacteria *Scytonema* cf. *crispum* (97%, HM629429) and *Lyngbya wollei* (95%, EU603711.1). In contrast, the 128 bp sequence was most similar to the *sxtA* gene of *Aphanizomenon* (99%, HQ338481.1) and *Anabaena circinalis* (99%, HQ338478.1) (Supplemental Table 3). A phylogenetic tree (Fig. 3) was constructed using the 657 bp product of the *sxtA* gene in sample E, and the sequence grouped closest to the *Lyngbya wollei* (EU629174) and *Scytonema* (HM629429) sequence. One *Cylindrospermopsis* (EU629178) and several *Anabaena* and *Aphanizomenon* sequences clustered differently.

Table 1
Genes for toxin production in five Arctic cyanobacterial samples. Detection of the *mcy* and *sxt* operon in five Arctic cyanobacterial communities suggesting the potential for microcystin and saxitoxin production.

Sample	16S	*mcyA*	*mcyA*	*mcyB*	*mcyE*	PKS	*sxtA*	*sxtA*
A	+	+	+	–	+	+	–	–
B	+	+	–	+	–	–	–	–
C	+	+	–	–	+	–	–	–
D	+	+	+	+	–	+	–	–
E	+	+	+	+	+	+	+	+

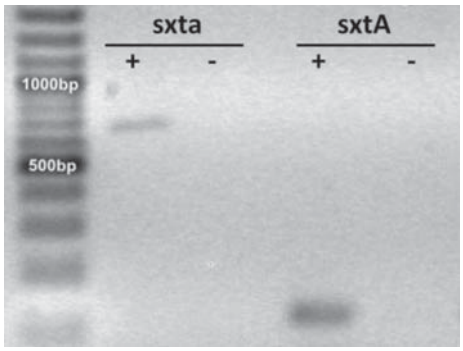


Fig. 2. mRNA expression of the *stxA* gene. Detection of mRNA expression of the *sxtA* gene in Arctic cyanobacterial sample A. Two different primer pairs sxta and sxtA were used resulting in a 650 bp and a 170 bp long product respectively. Negative controls were performed excluding reverse transcriptase to reduce false positive signals of DNA contamination.

3.1.2. Microcystin detection

Liquid chromatography MS analysis of sample A (Fig. 4A) that contained 106 μg MC/kg dry weight in the ELISA assay, showed that multiple MC variants were present in the extract. One of these compounds had a similar retention time to that of MC RR (Fig. 4B), but yielded a doubly protonated ion with m/z 526.7. Tandem MS of the m/z 526.7 ion revealed fragment ions resembling a diarginated MC (minus CN_2H_2; [35]). Also observed in the fragment ions was a loss of 60 Da which suggested that the MC contained ADMADDA instead of the generally observed ADDA (minus HOAc; [36]). Assignment of the fragment ions indicated that this MC also contained alanine (Ala), arginine (Arg), aspartic acid (Asp), glutamic acid (Glu) and an 83 Da moiety (Mdha or Dhb) in the sequence Ala Arg Asp Arg ADMADDA Glu Mdha/Dhb (Table 2).

The amino acid Mdha is generally observed at position seven of MCs, however, the isometric Dhb has also been observed. Therefore, a simple thiol derivatization was used to discriminate between the two amino acids. A MC containing a terminal alkene, such as found in Mdha, will readily react with β mercaptoethanol under alkaline conditions. When Dhb is present, the reaction rate is hundreds of times slower [31]. The β mercaptoethanol derivatization of an Mdha containing MC (MC RR) progressed with a half life of 2.6 min. Derivatization of the MC present in Sample A progressed much more slowly ($t_{½}$ = 1579 min), which suggested that the MC in Sample A contained Dhb in position seven and thus appeared to be the same MC [Asp^3, $ADMADDA^5$, Dhb^7] MC RR (Fig. 5) described previously in *Nostoc* [37].

There were several minor compounds present in Sample A that showed some structural similarity to MCs, but could not be identified using the current sample due to insufficient individual compound quantity. The four other samples which previously tested negative for MC by ELISA [29] tested negative for MC by LC MS.

All five samples were tested for the presence of genes of the *mcy* gene cluster, responsible for MC synthesis. Three different genes

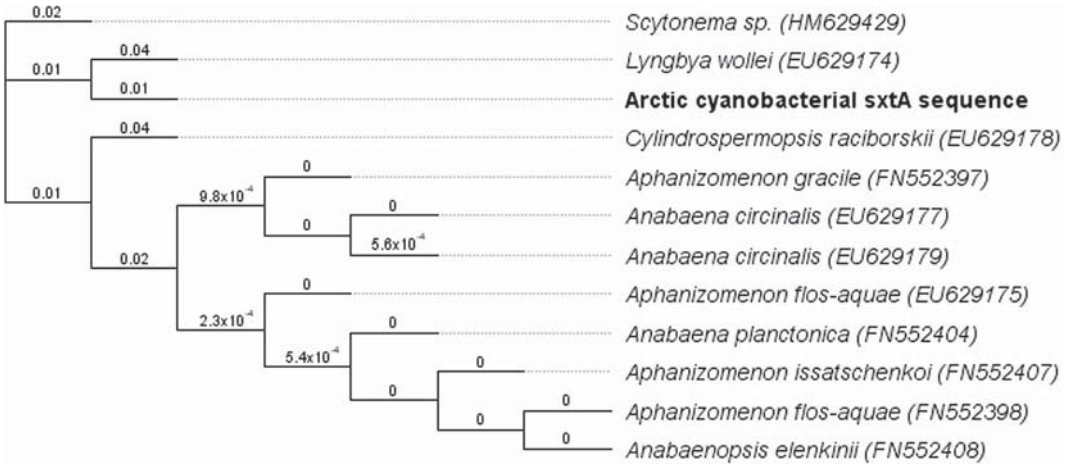


Fig. 3. Phylogenetic analysis of *sxtA*. The 657 bp long sequence obtained is given in boldface; other sequences were obtained from GenBank. Sequences were aligned and the tree constructed using Geneious™ software (version 5.5.6) as a Neighbour-Joining tree after the method of Jukes–Cantor. Substitutions per site are given next to the branches.

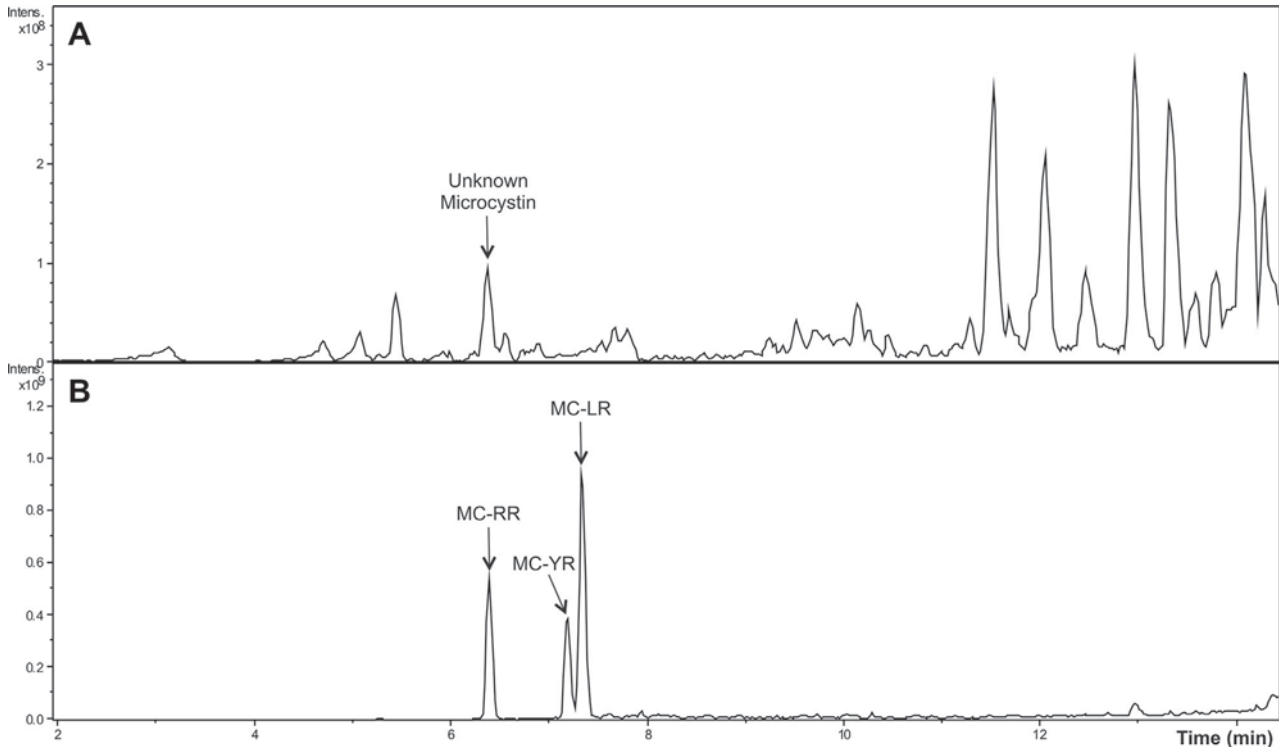


Fig. 4. Identification of the microcystin (MC) variant by liquid chromatography–mass spectrometry (LC–MS). High pressure LC–MS basepeak chromatograms (*m*/*z* 100–2000) of a methanolic extract of Artic cyanobacterial Sample A (A) and of authentic standards of MC-RR, MC-YR and MC-LR (B) separated on a C_{18} column.

involved in MC synthesis (*mcyA*, *mcyE* and polyketide synthase re gions) were amplified for sample A, which had tested positive for MC in the ELISA and LC MS analysis, providing a product of the correct size (Table 1). Subsequent sequencing of the products and GenBank comparison however resulted in only one product anno tated to a gene involved in secondary metabolite production, i.e. an amino acid adenylation domain of *Clostridium* (Supplemental Ta ble 3). The other two products did not result in a specific identifi cation of an annotated gene.

For the other four samples (B E), negative for MC in the ELISA and LC MS analysis, at least one gene involved in MC synthesis was amplified, sequenced and annotated in GenBank to a known gene involved in MC synthesis (Supplemental Table 3) with simi larities ranging between 60% and 99%. In total, seven sequences, annotated to genes involved in MC synthesis, were amplified. The genera corresponding to these genes were *Microcystis*, *Nostoc* and *Microcoleus* (Supplemental Table 3).

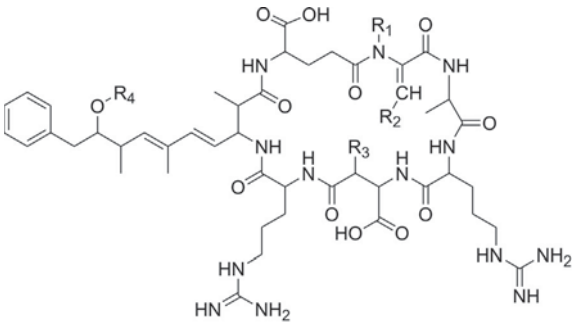
3.2. Potential toxin producers in Arctic cyanobacterial communities

A clone library was constructed for the samples that contained either MC (sample A) or STX (sample E) to identify the potential toxin producers. Genera were also identified using light micros copy. Molecular characterization of the 16S ITS region demon strated that the species present in the STX containing sample E were most similar to: *Nostoc puntiforme* (CP001037, 95%), *Lep tolyngbya* (*frigida*) (AY493573, 97%), *Calothrix* sp. (JN385289, 92%), *Snowella littoralis* (AJ781040, 98%), and *Tolypothrix distorta*

Table 2
Identification of the microcystin (MC) variant by LC-MS. Fragment-ion pattern detected for [Asp^3, ADMADDA^5, Dhb^7] MC-RR when observed by electrospray ionization collision-induced dissociation.

$[M + H]^{+a}$	$[M + 2H]^{2+}$	Fragment ion assignment
155.0	–	Dhb-Ala
157.1	–	Arg
213.0	–	Glu-Dhb
265.1	–	ADMADDA-HOAc-NH_3
272.1	–	Asp-Arg
311.1	–	Dhb-Ala-Arg
426.2	–	Dhb-Ala-Arg-Asp
582.2	291.6	Dhb-Ala-Arg-Asp-Arg
613.2	–	Asp-Arg-ADMADDA
627.2	–	Arg-ADMADDA-Glu
710.3	–	Arg-ADMADDA-Glu-Dhb
711.3	–	Glu-Dhb-Ala-Arg-Asp-Arg
742.2	–	Asp-Arg-ADMADDA-Glu
937.4	469.2	M – Asp
981.4	491.2	M – Ala
992.4	496.7	M – HOAc
1008.2	504.6	M – CN_2H_2
1034.4	517.7	M – H_2O
1052.4	526.7	M

[a] *m*/*z* Values in italics are deconvoluted from their respective $[M + 2H]^{2+}$ ions.



Microcystin (MC)	R_1	R_2	R_3	R_4
MC-RR	$-CH_3$	-H	$-CH_3$	$-CH_3$
[Asp^3,ADMADDA^5, Dhb^7] MC-RR	-H	$-CH_3$	-H	$-COCH_3$

Fig. 5. Chemical structure of detected and reference microcystins (MCs). Structures of MC-RR and [Asp^3, ADMADDA^5, Dhb^7] MC-RR.

(GQ287651, 98%), see Supplemental Table 4. Fewer cyanobacterial signals were obtained for sample A, and the sequences were most similar to; *Aphanizomenon gracile* (FJ424575, 94%), *Leptolyngbya* sp. (DQ431004, 94%), and *Chroococcus* (FR798926, 97%). As a general guideline strains with >97% 16S rRNA gene sequence similarity are considered to belong to the same species, so that annotations based on lower values as described for some of the strains here need to be handled with care. Light microscopy showed that both samples had a similar appearance, with dominating Nostocales embedded in a firm mucilaginous matrix. Other orders, albeit in lower abundance, i.e. Oscillatoriales (e.g. *Leptolyngbya*) and Chroococcales were also present. The Nostocales observed had cells of 3–6 μm in diameter and contained heterocytes (Supplemental Fig. 2). *Leptolyngbya* with a trichome width of approximately 1.7 μm was present in both samples. In Sample A *Tolypothrix* was characterized by dark brown colored sheaths and a trichome width of approximately 15 μm.

4. Discussion

Cyanobacterial toxin production is a worldwide phenomenon with concomitant widespread adverse health effects in humans and wildlife of the temperate and tropical regions [38,39]. The adverse effects are not only of acute nature but can also entail fundamental changes to whole ecosystems upon chronic or intermittent acute exposure events [40,41]. Despite the high abundance of cyanobacteria in the Arctic [2], at present there is only a single recent report of MCs in Arctic cyanobacteria [29] and in cyanobacteria associated lichen [42]. Although MC was below the limit of detection in most of the environmental samples analyzed by Kleinteich et al. [29], MC concentrations increased dramatically when cultured under laboratory conditions and at higher ambient temperatures. This was also associated with a profound change in the species diversity of the cyanobacterial mats investigated. Whether the higher toxin quantities produced and/or the higher temperatures induced the change in species diversity could not be determined. Nor was it ascertained whether the higher toxin production was associated with a reallocation of energy investment either as an advantageous trait of toxin producing cyanobacteria or as the result of changing temperature stress. Irrespective of the latter, these data suggest that continued climatic change may led to increases in cyanotoxins in polar regions. In an extension in this study, the same cyanobacterial communities from the Canadian Arctic were analyzed for the presence of STX and further characterization of the MC was undertaken.

4.1. Saxitoxins in Arctic cyanobacteria and their potential producers

The presence of STX was confirmed for the first time in a polar freshwater environment via immunological analyses as well as the detection of part of the *sxt* gene cluster and the corresponding mRNA product. The levels detected (21 μg STX/kg dry weight) were much lower than those reported for pure cultures of benthic cyanobacteria of temperate regions (*Scytonema* cf. *crispum*, 66×10^6 μg STX/kg dry weight [30]; *Lyngbya wollei*, maximum 58×10^6 μg STX/kg dry weight [43]). The latter difference is likely a result of the low abundance of the producer in a mixed environmental sample and low nutrient availability from the ambient melt waters [44,45] in contrast to a highly nutrient supplemented culture medium. Lack of STX detection using HPLC FLD may be explained by the fact that approximately 26 different variants of STXs are known to date [46]. HPLC analysis relies on the detection of each individual STX variant. In contrast, multiple STX variants can be detected with the ELISA, albeit with different affinities according to the manufacturer (e.g. decarbamoyl STX with 29% and GTX 2 & 3 with 23%), thus giving a sum value of STXs present in the respective sample analyzed. Thus, if multiple variants are present in a sample, reliable HPLC detection depends upon the concentration level of each variant present and not on the total STX concentration (as determined by STX ELISA).

In contrast to MC which is widely distributed among cyanobacterial taxonomic groups [12], only a few cyanobacterial genera have been reported to produce the STXs, including *Aphanizomenon*, *Planktothrix* (unconfirmed), *Scytonema* and *Lyngbya* [30]. The limited number of STX producing cyanobacteria may explain why STXs and the *sxt* genes were detected only in one out of five Arctic cyanobacterial samples analyzed. Consequently, the distribution of STX producing species in the Arctic, at least in the area studied, could be limited and solely dependent on the unique species composition of the respective cyanobacterial community. Although the STX producing species could not be identified, *Scytonema* or *Lyngbya* appeared likely candidates. The latter genus seems to have a widespread distribution in the Arctic regions as it has been

reported from the Canadian High Arctic [47,48], as well as Spitsbergen [49]. The *sxtA* sequence obtained from this sample showed highest sequence similarity to *Scytonema* sequences. *Tolypotrix* or *Scytonema* were identified in the sample by microscopy and *Tolypothrix* had the closest match in the 16S rRNA gene sequence library. *Tolypothrix* and *Scytonema* both belong to the order of *Nostocales* and *Scytonema* has recently been identified as a STX producer [30].

4.2. Microcystins in Arctic cyanobacterial communities and their potential producers

In a previous study MC was detected in one of the five cyanobacterial samples (Sample A) using an ADDA specific ELISA assay [29]. In the present study the MC congener in Sample A was identified as [Asp^3, ADMADDA^5, Dhb^7] MC RR. Although there are no studies available on the cross reactivity of ADMADDA with the ADDA specific antibody of the ADDA ELISA kit, it is possible that the concentration of the ADMADDA substituted MC variant is underestimated in this assay. The [Asp^3, ADMADDA^5, Dhb^7] MC RR variant (Fig. 5) has been reported before by Beattie et al. [37] in a *Nostoc* strain (DUN901) isolated from brackish water in the United Kingdom and is reported here for the first time in the Arctic region. A MC with the same mass, [Asp^3, ADMADDA^5] MC RR, was recently reported in lichen (*Peltigera membranacea*/*Peltigera hymenia*) associated cyanobacteria (most likely *Nostoc* or *Nodularia*) from Scotland [42]. While Kaasalainen et al. reported the presence of [Asp^3, ADMADDA^5] MC RR, the characterization of the MC congeners identified was not reported. As MS/MS alone cannot discriminate between Mdha and Dhb, it is possible that the MC identified in their study could have also contained a Dhb moiety, as described in Fig. 5.

MCs have been reported previously in a range of habitats in the continental Antarctic (McMurdo Ice Shelf, Bratina Island and Dry Valleys; [26 28]). The MC analyses demonstrated the presence of new and unusual MC congeners, e.g. [Gly^1] MC LR and RR, some of which contained the acetyldesmethyl ADDA [ADMADDA^5] substitution [27] also observed in this study. Wood et al. identified the cyanobacterial genus *Nostoc* as an Antarctic MC producer [27] based on microscopy and molecular methods. The latter corroborated the reports by Beattie et al. and Kaasalainen et al. [37,42] suggesting that *Nostoc* could be the producer of the [Asp^3, ADMADDA^5, Dhb^7] MC RR congener (Fig. 5). Even though in this study all of the Arctic cyanobacterial communities were dominated by Nostocales, other potential MC producing genera of cyanobacteria were observed, e.g. *Leptolyngbya* [51]. Both *Nostoc* and *Leptolyngbya* were detected via genetic and microscopic analyses in samples A and E. Due to the unusual MC congener identified and the information provided by earlier publications [37,42,50], it is hypothesized that *Nostoc* could also be the MC producer in this Arctic sample. However, an exact identification of the MC producer is not possible based on the currently available data and attempts at sub culturing the producer remained unsuccessful to date.

Genetic analyses of the Arctic cyanobacterial samples revealed that at least some species within the communities may have the potential to produce MCs. All of the samples contained at least one gene of the *mcy* gene cluster, involved in MC production (Table 1; Supplemental Table 3). The genes detected, included the *mcyA*, the *mcyB* and, in one case, the *mcyE* region (Table 1, Supplemental Fig. 1), encoding for non ribosomal peptide synthetases and a hybrid enzyme, respectively [52]. The signal intensity of *mcyA* was distinctly higher in sample A which tested positive for MC, suggesting a high abundance of the MC producer. When compared to the GenBank database however, some of the sequence similarities to known *mcy* genes were low (60 99%; Supplemental Table 3). In general the cyanobacterial *mcy* operon is known to be highly variable [53,54] with sequence similarities of the *mcy* genes between the two most studied MC producing species, *M. aeruginosa* and *Planktothrix agardhii*, only ranging between 53 and 86% depending on the gene region [53]. Low sequence similarities as detected in this study may therefore be a result of the high geographic and phylogenetic separation between known MC producing cyanobacterial species from temperate and tropical regions and the Arctic species of this study. More sequence data and pure cultures are required to confirm genetic variations and phylogenetic relationships.

4.3. Ecological implications of toxins in Arctic cyanobacterial communities

The concentrations of STX and MC detected (21 μg STX/kg dry weight and 106 μg MC/kg dry weight, [29]) are low when compared to those found in planktonic cyanobacterial blooms of temperate and tropical regions (up to 65.6×10^6 μg STX/kg dry weight, [30]; and up to 20×10^6 μg MC/kg dry weight, [55]) but are comparable to the levels detected in cyanobacterial mats of the Antarctic (1 15,900 μg MC/kg dry weight, [27]). Nevertheless, the presence of these compounds, albeit in low concentrations, could have a selective long term effect on the organisms living in and feeding on the cyanobacterial communities. Depending on the spatial distribution of the toxin producer and the toxin itself within the mat, as well as on the feeding strategy, microorganisms and metazoans may be differentially exposed to the toxins. Saxitoxin and MC containing cyanobacteria have been reported to be toxic to nematodes, crustaceans and rotifers [56 60]; these groups are also present in many cyanobacterial mats. Trophic interactions in these mat communities are currently poorly understood and the effects of the toxins on the organisms present are largely unknown. It has been suggested that rising temperatures could increase toxin concentrations in polar cyanobacterial communities [29,61] and thus exacerbate potential effects of toxins on metazoan organisms [57]. In the cryosphere many physiological processes happen just above a minimum threshold level [62] and energy consuming production of secondary metabolites e.g. MC and STX may be limited [29]. Kaebernick and Neilan [19] postulated that MC synthesis is increased under optimal temperature and growth conditions, which are speculated to be around 20 °C for polar cyanobacteria [63]. Thus a warmer climate as predicted for the Arctic in current climate change models [64] could elevate the temperature above the minimum threshold level for toxin synthesis, leading to an increased general metabolic activity and thus an increased level of toxin production [29,65,66].

Conflict of interest

None.

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